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(54) Title: HUMAN ANTIBODIES DERIVED FROM IMMUNIZED XENOMICE

(57) Abstract

Antibodies with fully human variable regions against a specific antigen can be prepared by administering the antigen to a transgenic animal which has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled. Various subsequent manipulations can be performed to obtain either antibodies *per se* or analogs thereof.

HUMAN ANTIBODIES DERIVED FROM IMMUNIZED XENOMICE

Technical Field

The invention relates to the field of immunology, and in particular to the production of antibodies. More specifically, it concerns producing such antibodies by a process which includes the step of immunizing a transgenic animal with an antigen to which antibodies are desired. The transgenic animal has been modified so as to produce human, as opposed to endogenous antibodies.

Background Art

PCT application WO 94/02602, published 3 February 1994 and incorporated herein by reference, describes in detail the production of transgenic nonhuman animals which are modified so as to produce antibodies with fully human variable regions rather than endogenous antibodies in response to antigenic challenge. Briefly, the endogenous loci encoding the light and heavy immunoglobulin chains are incapacitated in the transgenic hosts and loci encoding human heavy and light chain proteins are inserted into the genome. In general, the animal which provides all the desired modifications is obtained by cross-breeding intermediate animals containing fewer than the full complement of modifications. The preferred embodiment of the nonhuman animal described in the specification is a mouse. Thus, mice, specifically, are described which, when administered immunogens, produce antibodies with human variable regions, including fully human antibodies, rather than murine antibodies that are immunospecific for these antigens.

The availability of such transgenic animals makes possible new approaches to the production of fully human antibodies. Antibodies with various immunospecificities are desirable for therapeutic and diagnostic use. Those antibodies intended for human therapeutic and *in vivo* diagnostic use, in particular, have been problematic because prior art sources for such antibodies resulted in immunoglobulins bearing the characteristic structures of antibodies produced by nonhuman

immortalized. The immortalized B cells can be used directly as the source of human antibodies or, alternatively, the genes encoding the antibodies can be prepared from the immortalized B cells or from primary B cells of the blood or lymphoid tissue (spleen, tonsils, lymph nodes, bone marrow) of the immunized animal and expressed in recombinant hosts, with or without modification, to produce the immunoglobulin or its analogs. In addition, the genes encoding the repertoire of immunoglobulins produced by the immunized animal can be used to generate a library of immunoglobulins to permit screening for those variable regions which provide the desired affinity. Clones from the library which have the desired characteristics can then be used as a source of nucleotide sequences encoding the desired variable regions for further manipulation to generate antibodies or analogs with these characteristics using standard recombinant techniques.

In another aspect, the invention relates to an immortalized nonhuman B cell line derived from the above described animal. In still another aspect, the invention is directed to a recombinant host cell which is modified to contain the gene encoding either the human immunoglobulin with the desired specificity, or an analog thereof which exhibits the same specificity.

In still other aspects, the invention is directed to antibodies or antibody analogs prepared by the above described methods and to recombinant materials for their production.

In still other aspects, the invention is directed to antibodies with fully human variable regions, including fully human antibodies which are immunospecific with respect to particular antigens set forth herein and to analogs which are similarly immunospecific, as well as to the recombinant materials useful in the production of these antibodies.

Brief Description of the Drawings

Figure 1 shows the serum titers of anti-IL-6 antibodies from a Xenomouse™ immunized with human IL-6 and which antibodies contain human κ light chains and/or human μ heavy chains.

contain human heavy chain μ constant regions or human κ light chains.

Figure 12 shows the results of a FACS analysis of antibodies from a Xenomouse™ (labeled A247-4) immunized with human gp39 reacted with activated human T cells. Figure 12A shows the separation of human activated T cells into $CD4^+$ and $CD4^-$ populations. Panel B shows the results of a FACS analysis of the activated $CD4^+$ T cells with antibodies from the Xenomouse™ immunized with gp39 which contain murine heavy chain γ constant regions; panel C shows the corresponding results with respect to $CD4^-$ populations.

Figure 13 is a titration curve with respect to monoclonal antibodies secreted by the hybridoma clone D5.1. This clone is obtained from a Xenomouse™ immunized with tetanus toxin C (TTC) and contains human κ light chain and human μ constant region in the heavy chain.

Figure 14 is a titration curve with respect to the hybridoma supernatant from clone K4.1. This hybridoma clone is obtained from a Xenomouse™ immunized with TTC and contains human κ light chain and heavy chain having the murine γ constant region.

Figure 15 shows binding curves for various concentrations of the K4.1 monoclonal antibody in a determination of the affinity of the monoclonal with its antigen in a BIAcore instrument.

Figure 16 shows the complete nucleotide sequence of the heavy chain from the antibody secreted by K4.1.

Figure 17 shows the complete nucleotide sequence of the light chain from the antibody secreted by K4.1.

Figure 18 shows the complete nucleotide sequence of the heavy chain from the antibody secreted by D5.1.

Figure 19 shows the complete nucleotide sequence of the light chain from the antibody secreted by D5.1.

Modes of Carrying Out the Invention

In general, the methods of the invention include administering an antigen for which human forms of immunospecific reagents are desired to a transgenic nonhuman animal which has

(1991) 9:____. The construction of antibody analogs with multiple immunospecificities is also possible by coupling the human variable regions derived from antibodies with varying specificities.

The variable regions with fully human characteristics can also be coupled to a variety of additional substances which can provide toxicity, biological functionality, alternative binding specificities and the like. The moieties including the fully human variable regions produced by the methods of the invention include single-chain fusion proteins, molecules coupled by covalent methods other than those involving peptide linkages, and aggregated molecules. Examples of analogs which include variable regions coupled to additional molecules covalently or noncovalently include those in the following nonlimiting illustrative list. Traunecker, A. *et al.* Int J Cancer Supp (1992) Supp 7:51-52 describe the bispecific reagent janusin in which the F_V region directed to CD3 is coupled to soluble CD4 or to other ligands such as OVCA and IL-7. Similarly, the fully human variable regions produced by the method of the invention can be constructed into F_V molecules and coupled to alternative ligands such as those illustrated in the cited article. Higgins, P.J. *et al.* J Infect Disease (1992) 166:198-202 describe a heteroconjugate antibody composed of OKT3 cross-linked to an antibody directed to a specific sequence in the V3 region or GP120. Such heteroconjugate antibodies can also be constructed using at least the human variable regions contained in the immunoglobulins produced by the invention methods. Additional examples of bispecific antibodies include those described by Fanger, M.W. *et al.* Cancer Treat Res (1993) 68:181-194 and by Fanger, M.W. *et al.* Crit Rev Immunol (1992) 12:101-124. Conjugates that are immunotoxins including conventional antibodies have been widely described in the art. The toxins may be coupled to the antibodies by conventional coupling techniques or immunotoxins containing protein toxin portions can be produced as fusion proteins. The analogs of the present invention can be used in a corresponding way to obtain such immunotoxins. Illustrative of such immunotoxins are those

chromatography with respect to the particular antigen, or even with respect to the particular epitope of the antigen for which specificity is desired. In any case, in order to monitor the success of immunization, the antibody levels with respect to the antigen in serum will be monitored using standard techniques such as ELISA, RIA and the like.

It will be noted, from the examples below, that a portion of the polyclonal antiserum obtained may include an endogenous heavy chain constant region derived from the host, even though the variable regions are fully human. Under these circumstances, to the extent that an application requires fully human antibodies, use of the polyclonal antiserum directly would be inappropriate. However, the presence of these chimeras, which is believed to result from *in vivo* isotype switching as described by Gerstein *et al.* Cell (1990) 63:537, is not problematic, in view of conventional purification and modification methods and in view of the availability of alternative methods to recover fully human antibodies, if desired, described in the following paragraphs.

First, and most simply, the polyclonal antiserum could be subjected to suitable separation techniques to provide compositions containing only fully human immunoglobulins. Portions of the serum which display characteristics of the host species can be removed, for example, using affinity reagents with the appropriate anti species immunoglobulins or immunospecific portions thereof. Furthermore, for applications where only the variable regions of the antibodies are required, treating the polyclonal antiserum with suitable reagents so as to generate F_{ab}, $F_{ab'}$, or $F_{(ab')_2}$ portions results in compositions containing fully human characteristics. Such fragments are sufficient for use, for example, in immunodiagnostic procedures involving coupling the immunospecific portions of immunoglobulins to detecting reagents such as radioisotopes. Thus, for some applications, the polyclonal antiserum can be treated to provide compositions with the desired characteristics including compositions consisting essentially of fully human antibodies and compositions including immunoglobulin analogs wherein the immunospecific portion is fully human.

As an alternative to obtaining human immunoglobulins directly from the culture of immortalized B cells derived from the animal, the immortalized cells can be used as a source of rearranged heavy chain and light chain loci for subsequent expression and/or genetic manipulation. Isolation of genes from such antibody-producing cells is straightforward since high levels of the appropriate mRNAs are available for production of a cDNA library. The recovered rearranged loci can be manipulated as desired. For example, the constant region can be exchanged for that of a different isotype or that of a human antibody, as described above, or eliminated altogether. The variable regions can be linked to encode single chain F_V regions. Multiple F_V regions can be linked to confer binding ability to more than one target or chimeric heavy and light chain combinations can be employed. Once the genetic material is available, design of analogs as described above which retain their ability to bind the desired target, as well as their human characteristics, is straightforward.

Once the appropriate genetic material is obtained and, if desired, modified to encode an analog, the coding sequences including those that encode, at a minimum, the variable regions of the human heavy and light chain can be inserted into expression systems contained on vectors which can be transfected into standard recombinant host cells. As described below, a variety of such host cells may be used; for efficient processing, however, mammalian cells are preferred. Typical mammalian cell lines useful for this purpose include CHO cells, 293 cells, or NSO-GS cells.

The production of the antibody or analog is then undertaken by culturing the modified recombinant host under culture conditions appropriate for the growth of the host cells and the expression of the coding sequences. The antibodies are then recovered from the culture. The expression systems are preferably designed to include signal peptides so that the resulting antibodies are secreted into the medium; however, intracellular production is also possible.

In addition to deliberate design of modified forms of the immunoglobulin genes to produce analogs, advantage can be

reagents with respect to antigens for which human antibodies have not heretofore been available. The immunoglobulins that result from the above-described methods and the analogs made possible thereby, provide novel compositions for use in analysis, diagnosis, research, and therapy. The particular use will, of course, depend on the immunoglobulin or analog prepared. In general, the compositions of the invention will have utilities similar to those ascribable to nonhuman antibodies directed against the same antigen. Such utilities include, for example, use as a affinity ligands for purification, as reagents in immunoassays, as components of immunoconjugates, and as therapeutic agents for appropriate indications.

Particularly in the case of therapeutic agents or diagnostic agents for use *in vivo*, it is highly advantageous to employ antibodies or their analogs with fully human characteristics. These reagents avoid the undesired immune responses engendered by antibodies or analogs which have characteristics marking them as originating from non-human species. Other attempts to "humanize" antibodies do not result in reagents with fully human characteristics. For example, chimeric antibodies with murine variable regions and human constant regions are easily prepared, but, of course, retain murine characteristics in the variable regions. Even the much more difficult procedure of "humanizing" the variable regions by manipulating the genes encoding the amino acid sequences that form the framework regions does not provide the desired result since the CDRs, typically of nonhuman origin, cannot be manipulated without destroying immunospecificity. Thus, the methods of the present invention provide, for the first time, immunoglobulins that are fully human or analogs which contain immunospecific regions with fully human characteristics.

There are large numbers of antigens for which human antibodies and their human analogs would be made available by the methods of the invention. These include the following as a nonlimiting set:

leukocyte markers, such as CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD11a,b,c, CD13, CD14, CD18, CD19, CD20, CD22, CD23,

toxins, such as pseudomonas endotoxin and osteopontin/uropontin, snake venom, and bee venom;

blood factors, such as complement C3b, complement C5a, complement C5b-9, Rh factor, fibrinogen, fibrin, and myelin associated growth inhibitor;

enzymes, such as cholesterol ester transfer protein, membrane bound matrix metalloproteases, and glutamic acid decarboxylase (GAD); and

miscellaneous antigens including ganglioside GD3, ganglioside GM2, LMP1, LMP2, eosinophil major basic protein, eosinophil cationic protein, pANCA, Amadori protein, Type IV collagen, glycated lipids, γ-interferon, A7, P-glycoprotein and Fas (AFO-1) and oxidized-LDL.

Particularly preferred immunoglobulins and analogs are those immunospecific with respect to human IL-6, human IL-8, human TNFα, human CD4, human L-selectin, and human gp39. Human antibodies against IL-8 are particularly useful in preventing tumor metastasis and inflammatory states such as asthma and reperfusion injury. Antibodies and analogs immunoreactive with human TNFα and human IL-6 are useful in treating cachexia and septic shock as well as autoimmune disease. Antibodies and analogs immunoreactive with gp39 or with L-selectin are also effective in treating or preventing autoimmune disease. In addition, anti-gp39 is helpful in treating graft versus host disease, in preventing organ transplant rejection, and in treating glomerulonephritis. Antibodies and analogs against L-selectin are useful in treating ischemia associated with reperfusion injury.

Typical autoimmune diseases which can be treated using the above-mentioned antibodies and analogs include systemic lupus erythematosus, rheumatoid arthritis, psoriasis, Sjogren's, scleroderma, mixed connective tissue disease, dermatomyositis, polymyositis, Reiter's syndrome, Behcet's disease, Type 1 diabetes, Hashimoto's thyroiditis, Grave's disease, multiple sclerosis, myasthenia gravis and pemphigus.

The examples below are intended to illustrate but not to limit the invention.

determined after the second dose and following each dose thereafter. Bleeds were performed 6-7 days after injections from the retrobulbar plexus. The blood was allowed to clot at room temperature for about 2 hours and then incubated at 4°C for at least 2 hours before separating and collecting the sera.

ELISAs were conducted as described above by applying 100 µl/well of recombinant human IL-6 at 2 mg/ml in coating buffer. Plates were then incubated at 4°C overnight or at 37°C for 2 hours and then washed three times in washing buffer. Addition of 100 µl/well blocking buffer was followed by incubation at room temperature for 2 hours, and an additional 3 washes.

Then, 50 µl/well of diluted serum samples (and positive and negative controls) were added to the plates. Plates were then incubated at room temperature for 2 hours and again washed 3 times.

After washing, 100 µl/well of either mouse antihuman µ chain antibody conjugated to HRP at 1/2,000 or mouse antihuman κ chain antibody conjugated to HRP at 1/2,000, diluted in blocking buffer were added. After a 1 hour incubation at room temperature, the plates were washed 3 times and developed with OPD substrate for 10-25 minutes. 50 µl/well of stop solution were then added and the results read on an ELISA plate reader at 492 nm. The dilution curves resulting from the titration of serum from Xenomouse™ A40-7 after 6 injections are shown in Figure 1. The data in Figure 1 show production of anti-IL-6 immunoreactive with antihuman κ and antihuman µ detectable at serum dilutions above 1:1,000.

Example 2

Human Antibodies Against Human IL-8

Immunization and serum preparation were as described in Example 1 as except that human recombinant IL-8 was used as an immunogen.

ELISA assays were performed with respect to the recovered serum, also exactly as described in Example 1, except that the ELISA plates were initially coated using 100 µl/well of recombinant human IL-8 at 0.5 mg/ml in the coating buffer. The

(Becton Dickinson). The cells were stained for human CD4 with a mouse antihuman CD4- PE antibody and the top 2-3% expressing cells were selected.

Immunizations were conducted as described in Example 1 using 10 x 10^6 cells per mouse except that the primary injection was subcutaneous at the base of the neck. The mice received 6 injections 2-3 weeks apart. Serum was prepared and analyzed by ELISA as described in Example 1 except that the initial coating of the ELISA plate utilized 100 µl per well of recombinant soluble CD4 at 2 mg/ml of coating buffer. The titration curve for serum from Xenomouse™ A207-1 after 6 injections is shown in Figure 4. Titers of human anti-CD4 reactivity were shown at concentrations representing greater than those at 1:1,000 dilution.

Example 5

Human Antibodies Against Human L-selectin

The antigen was prepared as a surface displayed protein in C51 cells, a high expressing clone derived by transfecting the mouse pre-B cell 300.19 with LAM-1 cDNA (LAM-1 is the gene encoding L-selectin) (Tedder, *et al.*, J Immunol (1990) 144:532) or with similarly transfected CHO cells. The transfected cells were sorted using fluorescent activated cell sorting using anti-Leu-8 antibody as label.

The C51 and the transfected CHO cells were grown in DME 4.5 g/l glucose with 10% FCS and 1 mg/ml G418 in 100 mm dishes. Negative control cells, 3T3-P317 (transfected with gag/pol/env genes of Moloney virus) were grown in the same medium without G418.

Primary immunization was done by injection subcutaneously at the base of the neck; subsequent injections were intraperitoneal. 70-100 million C51 or transfected CHO cells were used per injection for a total of five injections 2-3 weeks apart.

Sera were collected as described in Example 1 and analyzed by ELISA in a protocol similar to that set forth in Example 1.

L-selectin-Ig fusion protein. Detection employed HRP-mouse antihuman κ and HRP-goat antimouse IgG.

Figure 7 shows the results from Xenomouse™ A195-2; antibodies specific for L-selectin having human κ light chains and/or human variable regions with murine heavy chain γ regions are present in the serum.

The antisera obtained from the immunized xenomice were also tested for staining of human neutrophils which express L-selectin. Human neutrophils were prepared as follows: peripheral blood was collected from normal volunteers with 100 units/ml heparin. About 3.5 ml blood was layered over an equal volume of One-step Polymorph Gradient (Accurate Chemical, Westbury, NY) and spun for 30 minutes at 450 x g at 20°C. The neutrophil fraction was removed and washed twice in DPBS/2% FBS.

The neutrophils were then stained with either:

(1) antiserum from Xenomouse™ A195-2 immunized with C51 cells (expressing L-selectin);

(2) as a positive control, mouse monoclonal antibody LAM1-3 (against L-selectin); and

(3) as negative control, antiserum from a Xenomouse™ immunized with cells expressing human gp39.

The stained, washed neutrophils were analyzed by FACS. The results for antiserum from Xenomouse™ A195-2 are shown in Figures 8 and 9.

These results show the presence of antibodies in immunized Xenomouse™ serum which contain fully human variable regions immunoreactive with L-selectin. The negative control antiserum from mice immunized with gp39 does not contain antibodies reactive against human neutrophils. Serum from A195-2 (immunized with L-selectin-expressing cells) contains antibodies binding to human neutrophils detectable with a goat antimouse IgG antibody (Figure 8), which binds with heavy chain protein composed of fully human variable regions and mouse γ constant regions. Staining with anti L-selectin Xenomouse™ antisera detected with a mouse monoclonal antibody against human κ chain antibody is shown in Figure 9, showing the presence of fully human κ light chain.

procedure was conducted substantially as set forth in Example 1; the microtiter plates were coated with CHO cells expressing gp39 grown in a 100 mm dish in DMEM, 4.5 g/l glucose, 10% FCS, 4 mM glutamine, and nonessential amino acid (NEAA) solution for MEM (100X). On the day preceding the ELISA assay, the cells were trypsinized and plated into 96-well filtration plates at 10^5 cells/200 μl well and incubated at 37°C overnight. The positive controls were mouse antihuman gp39; negative controls were antisera from mice immunized with an antigen other than gp39. 50 μl of sample were used for each assay. The remainder of the assay is as described in Example 1.

The dilution curves for the sera obtained after 4 injections from mice immunized with gp39 expressed on CHO cells are shown in Figure 11. As shown, the sera contained antihuman gp39 immunospecificity which is detectable with human κ and human μ chain antibodies coupled to HRP.

In addition, the sera were tested for their ability to react with activated human T cells included in PBMC using FACS analysis. To prepare the PBMC, human peripheral blood was collected from normal volunteers with the addition of 100 unit/ml heparin. PBMC were isolated over Ficoll gradient and activated with 3 μg/ml PHA, 1 μg/ml PMA in IMDM plus 10% FBS plus 25 μM 2-mercaptoethanol for 4 hours. After washing, the PBMC were stained with mouse Mab against human CD4 labeled with FITC to permit separation of $CD4^+$ and $CD4^-$ human T cells.

The activated $CD4^+$ and $CD4^-$ T cells were then analyzed by FACS using staining with either:

1) antiserum from a Xenomouse™ immunized with 300.19 cells producing gp39;

2) a positive control mouse Mab directed against α-CD40L (human gp39); and

3) a negative control antiserum from a Xenomouse™ immunized with TNF.

The detecting antibody in the FACS analysis was goat antimouse IgG (PE). The results are shown in Figure 12.

As shown in Figure 12A, $CD4^+$ (R2) and $CD4^-$ (R3) cells were separated prior to FACS analysis. Panel B shows the results for $CD4^+$ cells and shows that sera from mice immunized

used in addition to HRP mouse antihuman IgM as described in Example 1. Two hybridomas that secreted anti-TTC according to the ELISA assay, clone D5.1 and clone K4.1 were used for further analysis.

As shown in Figure 13, clone D5.1 secretes fully human anti-TTC which is detectable using HRP-conjugated antihuman μ chain antibody and HRP-conjugated antihuman κ chain antibody. This is confirmed in Figures 18 and 19. Figure 14 shows that clone K4.1 secretes anti-TTC which is immunoreactive with antimurine γ and antihuman κ HRP-conjugated antibodies. Thus, clone K4.1 provides anti-TTC fully with human variable region as confirmed in Figures 16 and 17 and a murine constant heavy chain γ region.

The antibodies secreted by D5.1 and K4.1 did not immunoreact in ELISAs using TNFα, IL-6, or IL-8 as immobilized antigen under conditions where positive controls (sera from xenomice immunized with TNFα, IL-6 and IL-8 respectively) showed positive ELISA results.

The affinity of the monoclonal antibodies secreted by K4.1 for TTC antigen was determined using commercially available reagents and instrumentation. BIAcore Instrument, CM5 sensor chips, surfactant P20 and the amine coupling kit were purchased from Pharmacia Biosensor (Piscataway, NJ). TTC was immobilized at two levels of antigen density on the surface of the sensor chips according to the manufacturer's instructions. Briefly, after washing and equilibrating the instrument with buffer containing surfactant, the surfaces were activated and the TCC was immobilized.

For high antigen density, the surface was activated with 35 μl of equal volumes 0.1 M NHS and 0.1 M EDC injected across the surface followed by 30 μl of TTC fragment at 100 μg/ml in 10 mM sodium acetate buffer pH 5.0. The surface was blocked by injecting 35 μl 1 M ethanolamine and washed to remove noncovalently bound TCC using 5 μl 0.1 M HCl. The entire immobilization procedure was conducted with a continuous flow of buffer at 5 μl/min. This results in about 7500-8500 response units (RU) of TTC per chip. (1000 RU corresponds to about 1 ng of protein per mm^2.)

Table 1

Kinetic Constants of K4.1 Measured Using the BIAcore on Two Different Surfaces

Immobilized tetanus toxinC	K4.1 conc. range nM	Association rate ka(10^5 $M^{-1}s^{-1}$)	Dissociation rate kd($10^5 s^{-1}$)	Binding constant KA(M^{-1}) =ka/kd	Dissociation constant KD(M) =kd/ka
931 RU	4.3 - 34.7	6.47 ± 1.05	4.02 ± 1.42	1.6 X 10^{10}	0.62 X 10^{-10}
868 RU	4.3 - 34.7	7.19 ± 2.18	2.02 ± 1.01	3.5 x 10^{10}	0.28 x 10^{-10}

Referring to Figure 17 which shows the light chain of the K4.1 antibody, analysis shows the presence of the human κ variable region B3 and joining region JK4. Eight nucleotides are missing from B3 at the V_K-J_K junction and four mutations were found in the variable region. Five nongermline nucleotide additions were present at the V_K-J_K junction.

Referring now to Figure 18 which sets forth the sequence for the heavy chain of the antibody secreted by clone D5.1, this shows the heavy chain is comprised of the human variable fragment VH6, the human diversity region DN1 and the human joining segment JH4 linked to the human μ constant region. There were two base-pair mutations from the germline sequence in the variable region, neither within the CDRs. Two additional mutations were in the D segment and six nongermline nucleotide additions were present at the D_H-J_H junction.

Finally, referring to Figure 19 which presents the light chain of the antibody secreted by D5.1, the human κ variable region B3 and human κ joining region JK3 are shown. There are nine base-pair differences from the germline sequences, three falling within CDR1.

Example 8

Production of Human Antibodies to IgE

A. Immunization of Mice

Germline chimeric mice containing integrated human DNA from the immunoglobulin loci were immunized by injection of 15-20 μg of human IgE/λ in adjuvant. The mice were boosted with 15-20 μg of human IgE/λ every 14 days after the primary immunization. A bleed was done on the immunized animals to test the titer of serum antibodies against human IgE/λ. The mice with the highest titers were sacrificed and the spleen removed.

B. Fusion of Splenocytes

Myeloma cells, line P3X63-Ag8.653, used as the fusion partner for the spleen cells, were thawed 6 days prior to the fusion and grown in tissue culture. One day before the fusion, cells were split into fresh medium containing 10% fetal calf serum (FCS) at a ratio of 1:3.

aliquots were stored in liquid nitrogen to preserve the cell stocks.

Using the foregoing procedures, antibodies specific for the antigens described above are prepared.

In accordance with the above procedure, mouse hybridomas producing human antibody against human IgE/λ were obtained.

In accordance with the above procedures, a chimeric nonhuman host, particularly a murine host, may be produced which can be immunized to produce human antibodies or analogs specific for an immunogen. In this manner, the problems associated with obtaining human monoclonal antibodies are avoided, because the transgenic host can be immunized with immunogens which could not be used with a human host. Furthermore, one can provide for booster injections and adjuvants which would not be permitted with a human host. The resulting B cells may then be used for immortalization for the continuous production of the desired antibody. The immortalized cells may be used for isolation of the genes encoding the immunoglobulin or analog and be subjected to further molecular modification by methods such as *in vitro* mutagenesis or other techniques to modify the properties of the antibodies. These modified genes may then be returned to the immortalized cells by transfection to provide for a continuous mammalian cellular source of the desired antibodies. The subject invention provides for a convenient source of human antibodies, where the human antibodies are produced in analogous manner to the production of antibodies in a human host. The animal host cells conveniently provide for the activation and rearrangement of human DNA in the host cells for production of human antibodies.

In accordance with the subject invention, human antibodies can be produced to human immunogens, e.g., proteins, by immunization of the subject host mammal with human immunogens. The resulting antisera will be specific for the human immunogen and may be harvested from the serum of the host. The immunized host B cells may be used for immortalization, e.g., myeloma cell fusion, transfection, etc. to provide immortal cells, e.g., hybridomas, to produce monoclonal

Claims

1. A method to produce an immunoglobulin having fully human variable region or an analog thereof, specific for a desired antigen, which method comprises:

administering said antigen or an immunogenic portion thereof to a nonhuman animal under conditions to stimulate an immune response, whereby said animal produces B cells that secrete immunoglobulin specific for said antigen; wherein said nonhuman animal is characterized by being substantially incapable of producing endogenous heavy and light immunoglobulin chain variable regions, but capable of producing human immunoglobulin variable regions; and

recovering said immunoglobulin or analog.

2. The method of claim 1 wherein said recovering step comprises recovering polyclonal immunoglobulin or analog from said animal.

3. The method of claim 1 wherein said recovering step comprises immortalizing B cells from said animal immunized with said antigen, screening the resulting immortalized cells for the secretion of said immunoglobulin specific for said antigen, and

1) recovering immunoglobulin secreted by said immortalized B cells, or

2) recovering the genes encoding at least the variable region of said immunoglobulin from the immortalized B cells, and optionally modifying said genes;

expressing said genes or modified forms thereof to produce immunoglobulin or analog; and

recovering said immunoglobulin or analog.

4. The method of claim 1 wherein said recovering step comprises

recovering genes encoding at least the variable region of immunoglobulins from the primary B cells of the animal immunized with said antigen;

administering a desired antigen or an immunogenic portion thereof to a nonhuman animal under conditions to stimulate an immune response, whereby said animal produces B cells that secrete immunoglobulin specific for said antigen; wherein said nonhuman animal is characterized by being substantially incapable of producing endogenous heavy and light immunoglobulin chain variable regions, but capable of producing human immunoglobulin variable regions;

recovering genes encoding at least the variable region of immunoglobulins from the primary B cells of the animal immunized with said antigen;

generating a library of said genes expressing the variable regions;

screening the library for a variable region with desired affinity for the antigen; and

recovering the genes encoding said variable regions and optionally modifying said genes.

9. The DNA molecule of claim 6, 7 or 8 wherein said encoding nucleotide sequence is operably linked to control sequences capable of effecting its expression.

10. A cell or cell line modified to contain the DNA molecule of claim 9.

11. A method to produce an immunoglobulin with fully human variable region or an analog thereof which method comprises culturing the cells of claim 10 under conditions whereby said encoding nucleotide sequence is expressed to produce said immunoglobulin or analog; and

recovering said immunoglobulin or analog.

12. An immortalized B cell which secretes an immunoglobulin with a fully human variable region to a desired antigen prepared by a method which comprises

administering said antigen or an immunogenic portion thereof to a nonhuman animal under conditions to stimulate an immune response, whereby said animal produces B cells that

18. The immunoglobulin or analog of claim 17 wherein the leukocyte marker is selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD11a,b,c, CD13, CD14, CD18, CD19, CD20, CD22, CD23, CD27 and its ligand, CD28 and its ligands B7.1, B7.2, B7.3, CD29 and its ligand, CD30 and its ligand, CD40 and its ligand gp39, CD44, CD45 and isoforms, CDw52 (Campath antigen), CD56, CD58, CD69, CD72, CTLA-4, LFA-1 and TCR;

the histocompatibility antigen is selected from the group consisting of MHC class I or II, the Lewis Y antigens, SLex, SLey, SLea, and SLeb;

the adhesion molecule is selected from the group consisting of VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, LFA-1, L-selectin, P-selectin, and E-selectin and their counterreceptors VCAM-1, ICAM-1, ICAM-2, LFA-3; Mac-1 and p150,95;

the interleukin is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, and IL-15;

the interleukin receptor is selected from the group consisting of IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-8R, IL-9R, IL-10R, IL-11R, IL-12R, IL-13R, IL-14R, and IL-15R;

the chemokine is selected from the group consisting of PF4, RANTES, MIP1α, MCP1, NAP-2, Groα, Groβ, and IL-8;

the growth factor is selected from the group consisting of TNFalpha, TGFbeta, TSH, VEGF/VPF, PTHrP, EGF family, FGF, PDGF family, endothelin, and gastrin releasing peptide (GRP);

the growth factor receptor is selected from the group consisting of TNFalphaR, RGFbetaR, TSHR, VEGFR/VPFR, FGFR, EGFR, PTHrPR, PDGFR family, EPO-R, GCSF-R and other hematopoietic receptors;

the interferon receptor is selected from the group consisting of IFNαR, IFNβR, and IFNγR;

the Ig and its receptor is selected from the group consisting of IgE, FceRI, and FCeRII;

the cell or cell line of claim 22 under conditions wherein said nucleotide sequence is expressed to produce said immunoglobulin or analog; and

recovering the immunoglobulin or analog.

24. An antibody containing a fully human variable region or analog thereof which is specifically immunoreactive with an antigen selected from the group consisting of transition state mimics; leukocyte markers; histocompatibility antigens; adhesion molecules; interleukins; interleukin receptors; chemokines; growth factors; growth factor receptors; interferon receptors; Igs and their receptors; tumor antigens; allergens; viral proteins; toxins; blood factors; enzymes; and the miscellaneous antigens ganglioside GD3, ganglioside GM2, LMP1, LMP2, eosinophil major basic protein, eosinophil cationic protein, pANCA, Amadori protein, Type IV collagen, glycated lipids, γ-interferon, A7, P-glycoprotein, Fas (AFO-1) and oxidized-LDL.

25. The antibody or analog of claim 24 wherein the leukocyte marker is selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD11a,b,c, CD13, CD14, CD18, CD19, CD20, CD22, CD23, CD27 and its ligand, CD28 and its ligands B7.1, B7.2, B7.3, CD29 and its ligand, CD30 and its ligand, CD40 and its ligand gp39, CD44, CD45 and isoforms, CDw52 (Campath antigen), CD56, CD58, CD69, CD72, CTLA-4, LFA-1 and TCR;

the histocompatibility antigen is selected from the group consisting of MHC class I or II, the Lewis Y antigens, SLex, SLey, SLea, and SLeb;

the adhesion molecule is selected from the group consisting of VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, LFA-1, L-selectin, P-selectin, and E-selectin and their counterreceptors VCAM-1, ICAM-1, ICAM-2, LFA-3; Mac-1 and p150,95;

the interleukin is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, and IL-15;

26. The antibody or analog of claim 24 wherein the desired antigen is selected from the group consisting of human IL-6, human IL-8, human TNFα, human CD4, human L-selectin, human gp39, human IgE and tetanus toxin C(TTC).

27. The antibody or analog of claim 19 or 26 wherein the desired antigen is human IL-6.

28. The antibody or analog of claim 19 or 26 wherein the desired antigen is human IL-8.

29. The antibody or analog of claim 19 or 26 wherein the desired antigen is human TNFα.

30. The antibody or analog of claim 19 or 26 wherein the desired antigen is human CD4.

31. The antibody or analog of claim 19 or 26 wherein the desired antigen is human L-selectin.

32. The antibody or analog of claim 19 or 26 wherein the desired antigen is human gp39.

33. The antibody or analog of claim 19 or 26 wherein the desired antigen is tetanus toxin C(TTC).

34. The antibody or analog of claim 19 or 26 wherein the desired antigen is human IgE.

35. The analog of claim 19 or 26 which is a single chain F_V.

36. The antibody or analog of claim 24 which is fully human.

37. The antibody or analog of claim 24 which is an agonist or is a catalyst or wherein the immunoglobulin is chimeric.

47. Use of the antibody of claim 27 for treating cachexia, septic shock, myeloma, renal cell carcinoma, osteoporosis, or Paget disease in a mammal.

48. Use of the antibody of claim 29 for treating septic shock, cachexia, osteoporosis, or systemic sclerosis in a mammal.

49. Use of the antibody of claim 28 for preventing tumor metastasis, and for treating asthma, rheumatoid arthritis, glomerulonephritis, reperfusion injury, adult respiratory distress syndrome, or systemic sclerosis in a mammal.

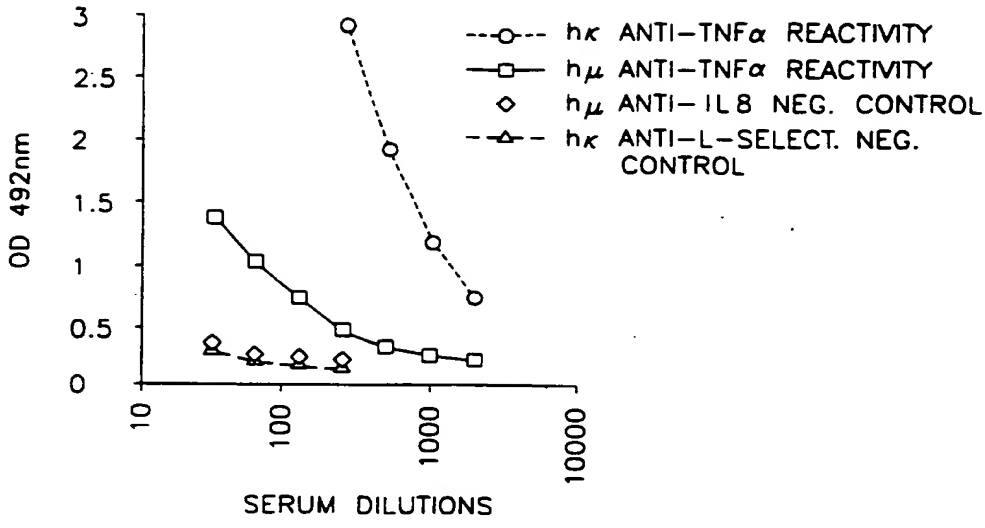
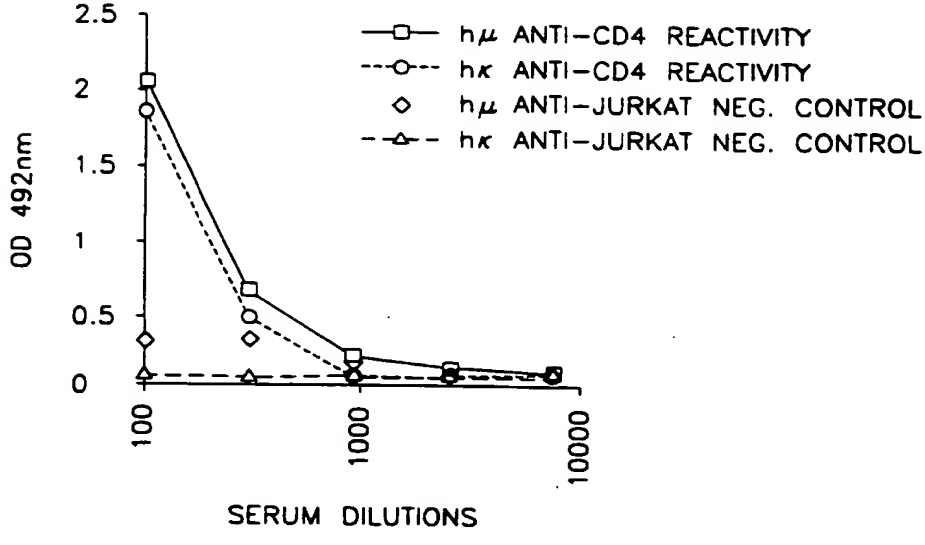

3
2.5
2
1.5
1
0.5
0
OD 492nm
10
100
1000
10000
SERUM DILUTIONS
hκ ANTI-TNFα REACTIVITY
hμ ANTI-TNFα REACTIVITY
hμ ANTI-IL8 NEG. CONTROL
hκ ANTI-L-SELECT. NEG. CONTROL

FIG. 3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2
1.5
1
0.5
0
OD 492nm
100
1000
10000
SERUM DILUTIONS
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hκ ANTI-CD4 REACTIVITY
hμ ANTI-JURKAT NEG. CONTROL
hκ ANTI-JURKAT NEG. CONTROL

FIG. 4

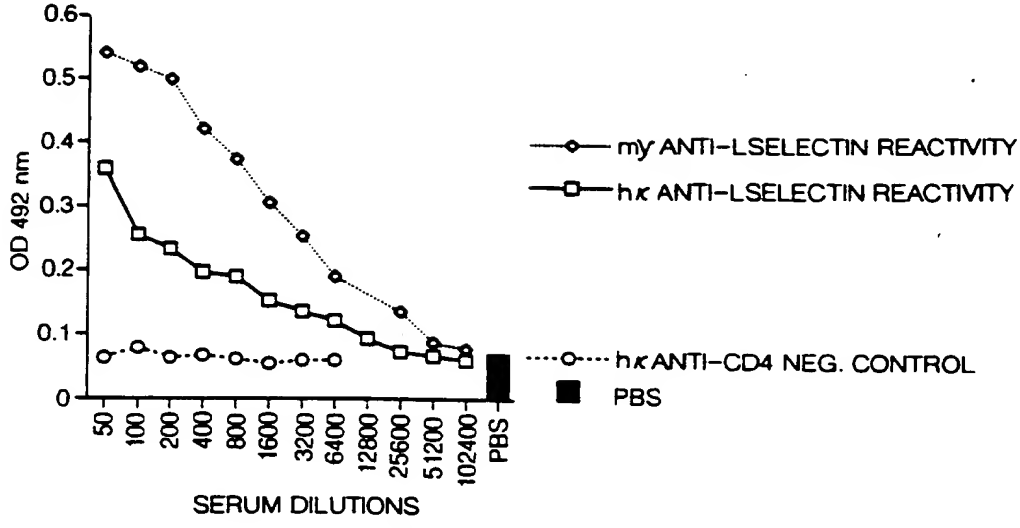

0.6
0.5
0.4
0.3
0.2
0.1
0
OD 492 nm
50
100
200
400
800
1600
3200
6400
12800
25600
51200
102400
PBS
SERUM DILUTIONS
mγ ANTI-LSELECTIN REACTIVITY
hκ ANTI-LSELECTIN REACTIVITY
hκ ANTI-CD4 NEG. CONTROL
PBS

FIG. 7

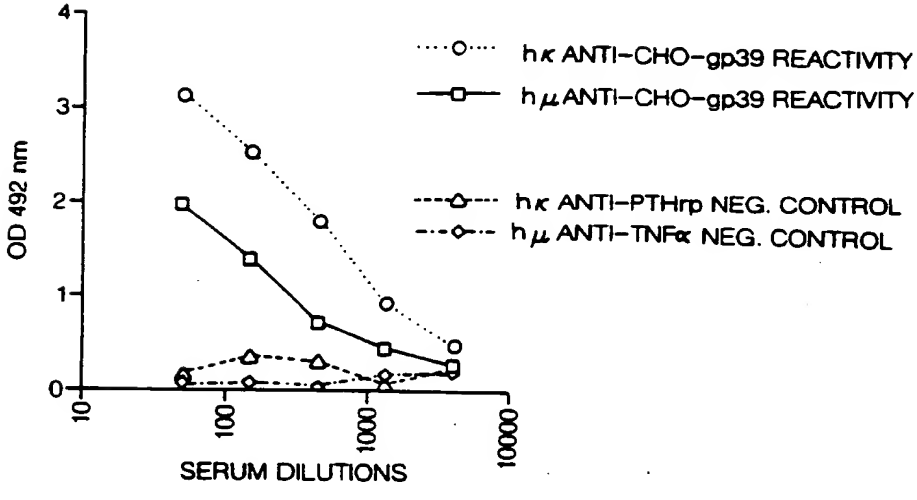
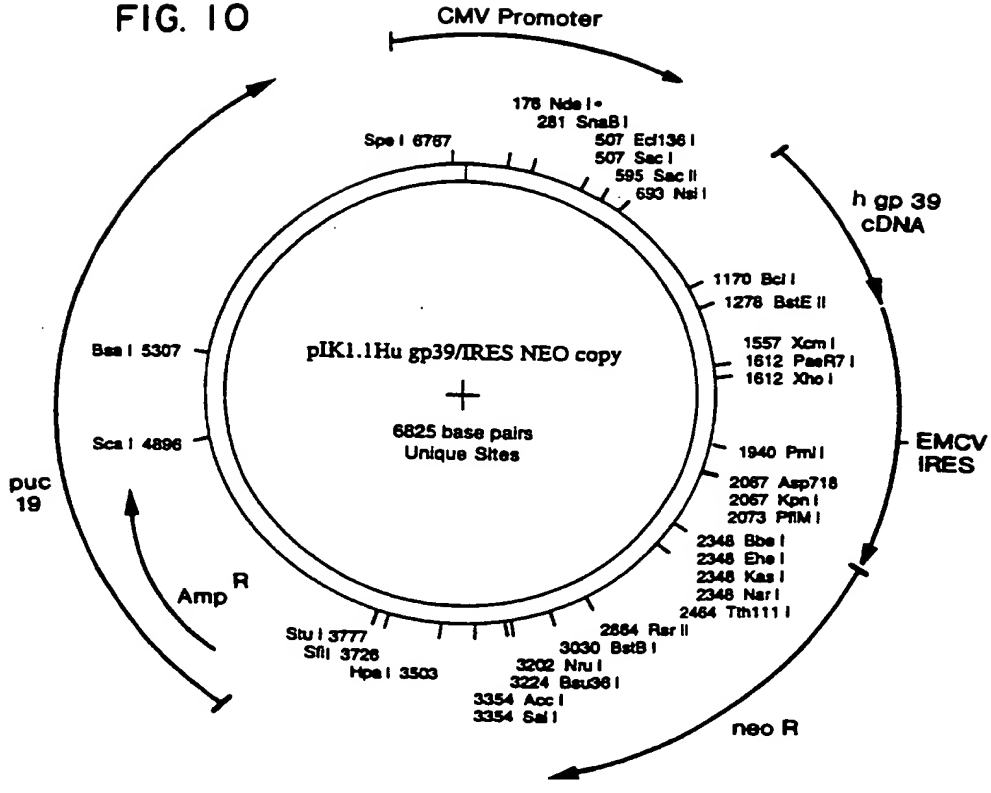

4
3
2
1
0
OD 492 nm
10
100
1000
10000
SERUM DILUTIONS
hκ ANTI-CHO-gp39 REACTIVITY
hμ ANTI-CHO-gp39 REACTIVITY
hκ ANTI-PTHrp NEG. CONTROL
hμ ANTI-TNFα NEG. CONTROL

FIG. 11

FIG. 10


CMV Promoter
178 Nde I
281 SnaB I
507 Ecl136 I
507 Sac I
595 Sac II
693 Nsi I
Spe I 6767
h gp 39 cDNA
1170 Bcl I
1278 BstE II
1557 Xcm I
1612 PaeR7 I
1612 Xho I
pIK1.1Hu gp39/IRES NEO copy
6825 base pairs
Unique Sites
Bsa I 5307
Sca I 4896
1940 Pml I
2067 Asp718
2067 Kpn I
2073 Pflm I
2348 Bbe I
2348 Ehe I
2348 Kas I
2348 Nar I
2464 Tth111 I
EMCV IRES
puc 19
Amp R
Stu I 3777
Sfil 3726
Hpa I 3503
2864 Rsr II
3030 BstB I
3202 Nru I
3224 Bsu36 I
3354 Acc I
3354 Sal I
neo R

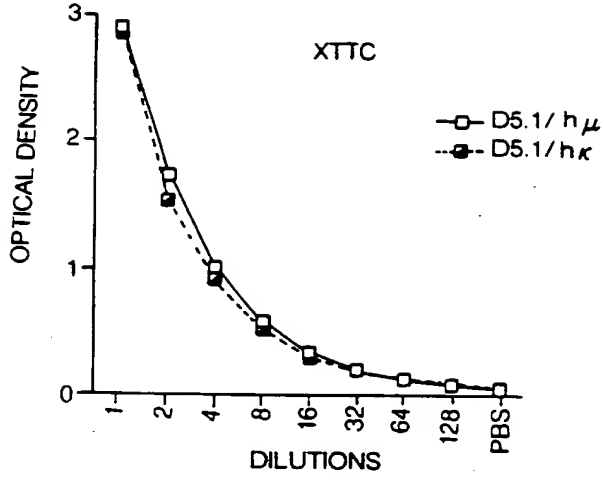
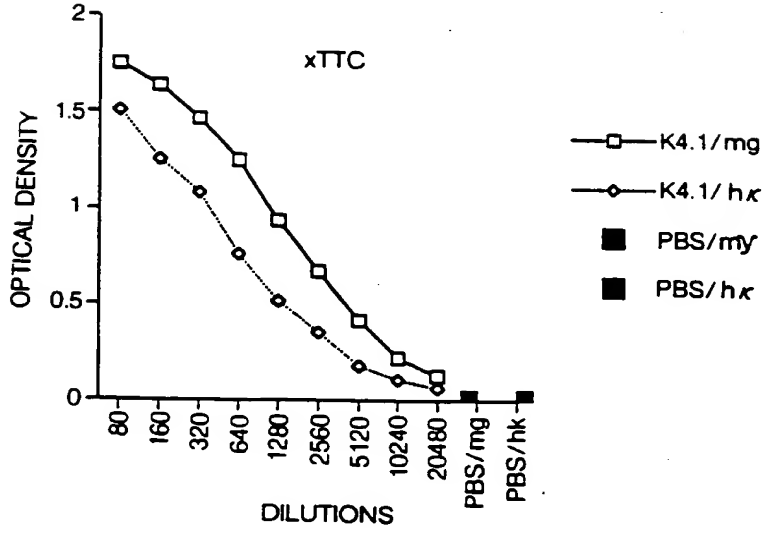

XTTC
OPTICAL DENSITY
3
2
1
0
D5.1/ hμ
D5.1/ hκ
1
2
4
8
16
32
64
128
PBS
DILUTIONS

FIG. 13


xTTC
OPTICAL DENSITY
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1.5
1
0.5
0
K4.1/mg
K4.1/ hκ
PBS/mγ
PBS/ hκ
80
160
320
640
1280
2560
5120
10240
20480
PBS/mg
PBS/hk
DILUTIONS

FIG. 14

SUBSTITUTE SHEET (RULE 26)

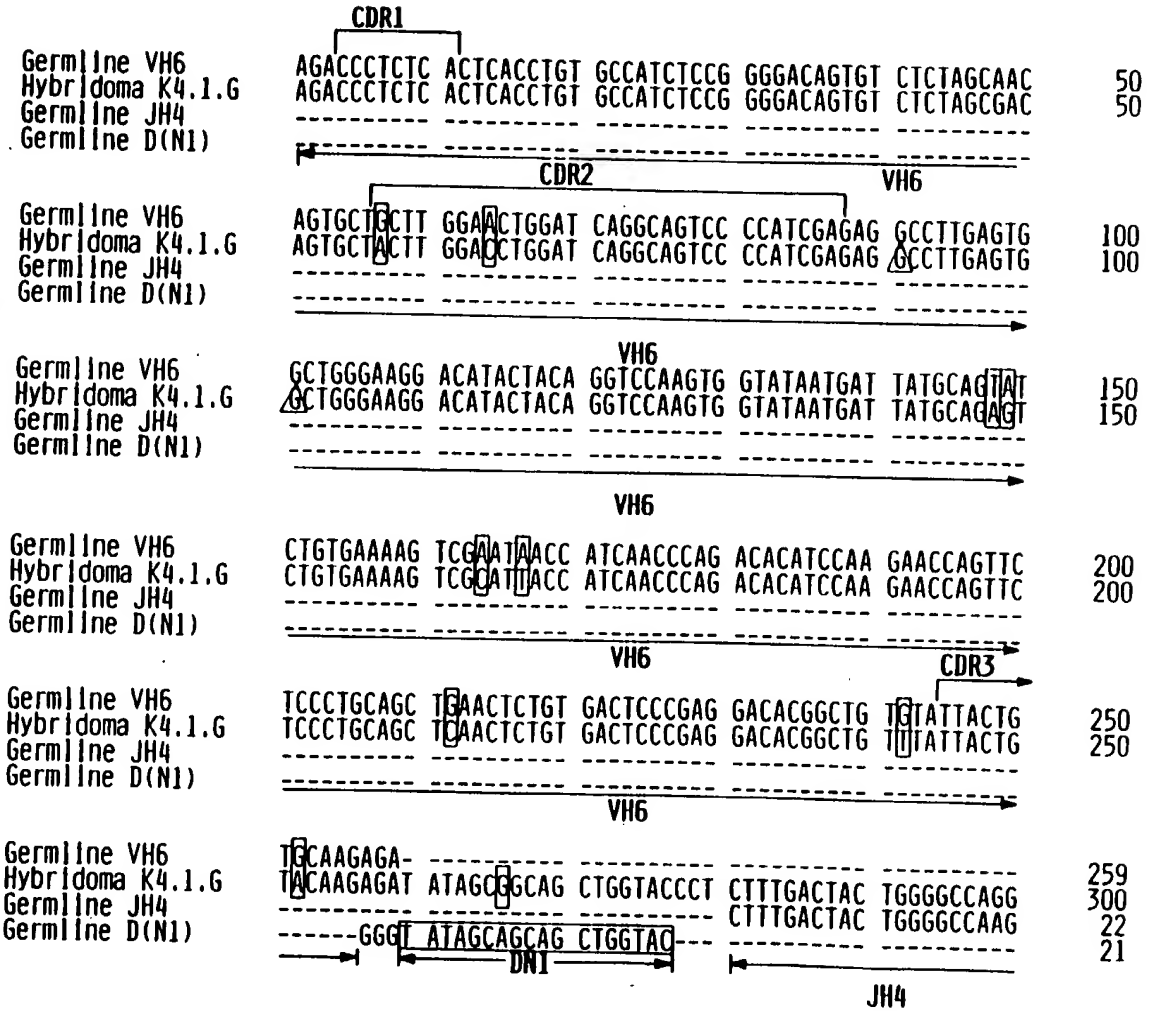

FIG. 16A

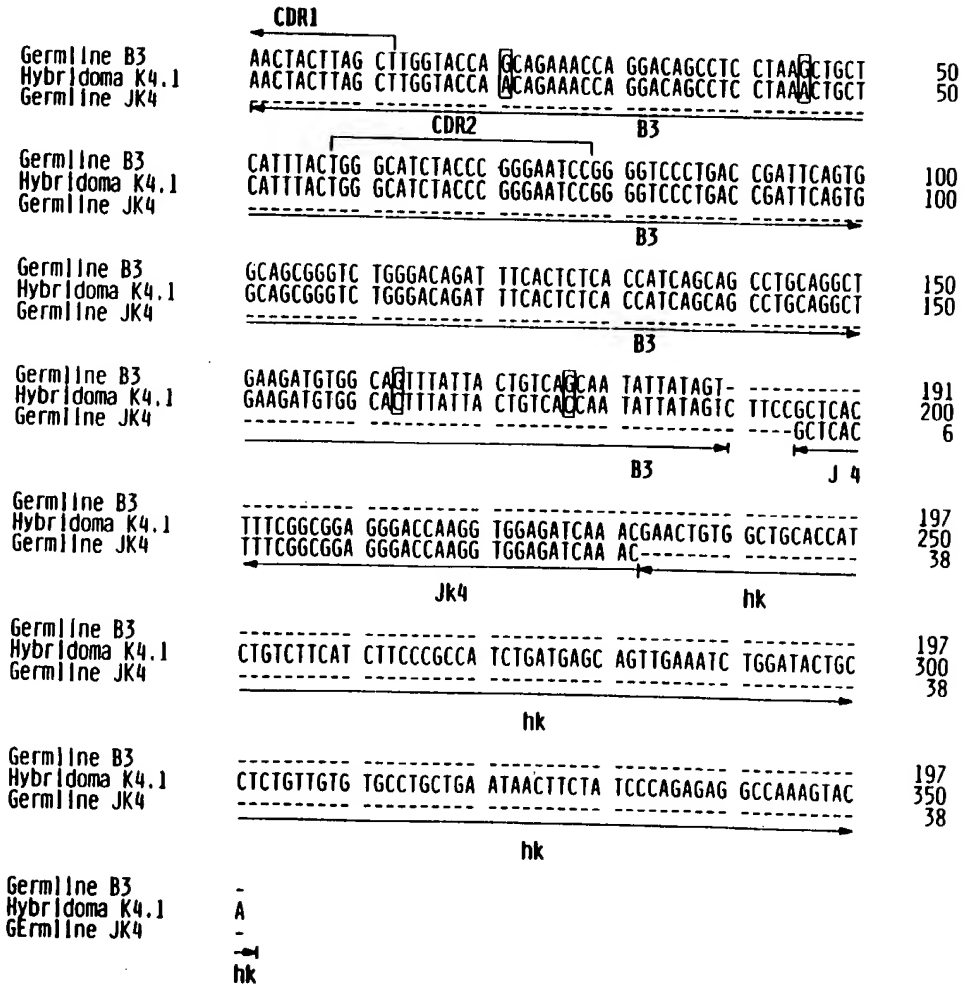

FIG. 17

SUBSTITUTE SHEET (RULE 26)

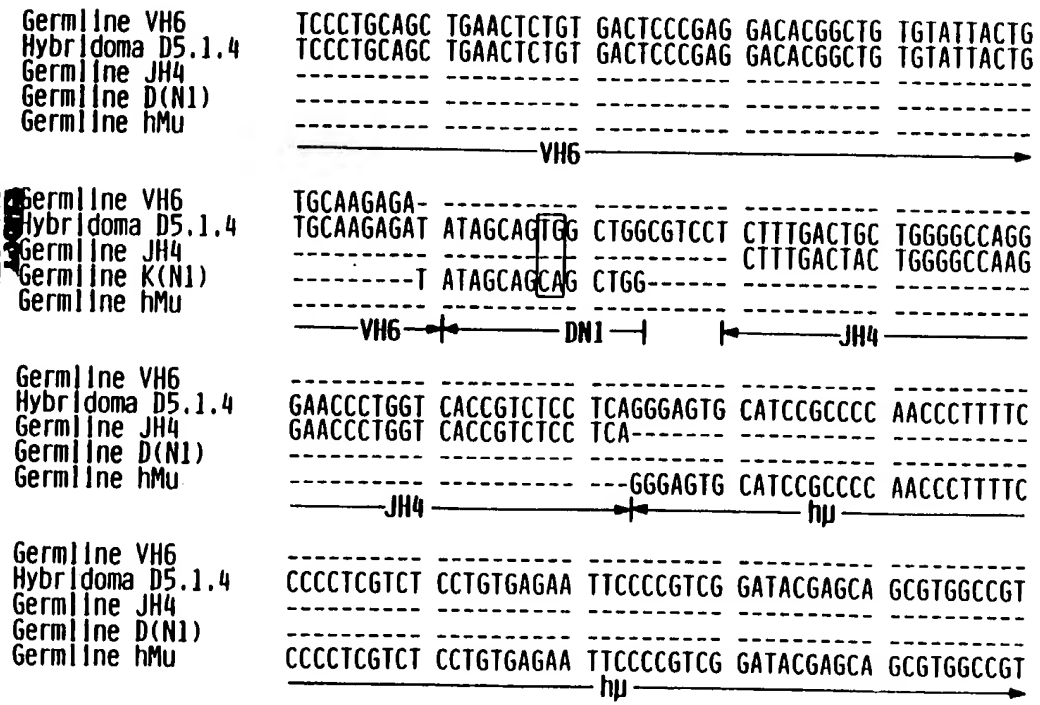

FIG. 18B

SUBSTITUTE SHEET (RULE 26)

```
Germline B3     TGCAGGCTGA AGATGTGGCA GTTTATTACT GTCAGCAATA TTATAGTACT
Hybridoma D5.1.4TGCAGGCTGA AGATGTGGCA GTTTATTACT GTCAGCAATA TTATAGTACT
Germline JK3    ---------- ---------- ---------- ---------- ----------
Germline CK     ---------- ---------- ---------- ---------- ----------
                ----------B3----------

Germline B3     CC-------- ---------- ---------- ---------- ----------
Hybridoma D5.1.4CCATTCAATT TCGGCCCTGG GACCAGAGTG GATATCAAAC GAACTGTGGC
Germline JK3    --ATTCACTT TCGGCCCTGG GACCAAAGTG GATATCAAAC ----------
Germline CK     ---------- ---------- ---------- GAACTGTGGC ----------
                ----------JK3----------CK----------

Germline B3     ---------- ---------- ---------- ---------- ----------
Hybridoma D5.1.4TGCACCATCT GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG
Germline JK3    ---------- ---------- ---------- ---------- ----------
Germline CK     TGCACCATCT GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG
                ----------CK----------

Germline B3     ---------- ---------- ---------- ---------- ----------
Hybridoma D5.1.4GAACTGCCTC TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC
Germline JK3    ---------- ---------- ---------- ---------- ----------
Germline CK     GAACTGCCTC TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC
                ----------CK----------

Germline B3     ---------- ---------- ---------- ---------- ----------
Hybridoma D5.1.4AAAGTACAGT GGAAGGTGGA TAACGCCCTC CAATCGGGTT GGGGAAAAA
Germline JK3    ---------- ---------- ---------- ---------- ----------
Germline CK     AAAGTACAGT GGAAGGTGGA TAACGCCCTC CAATCGGGT- ----------
                ----------CK----------
```

FIG. 19B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05500

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	European Journal of Immunology, Volume 21, issued 1991, (Bruggemann et al.), "Human antibody production in transgenic mice: expression from 100kb of the human IgH locus", pages 1323-1326, see entire article.	1-19, 24-37, 42
Y	Gene, Volume 87, issued 1990, (Lenz et al.), "Expression of heterobispecific antibodies by genes transferred into producer hybridoma cells", pages 213-218, see entire reference.	5-10
Y	Nature, volume 336, issued 24 November 1988, (Mansour et al.), "Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes", pages 348-352, see entire reference.	1-19, 24-37, 42
Y	EP, A, 0,315,062 (BRISTOL-MYERS COMPANY) 10 May 1989, see entire document.	1-19, 24-37, 42

INTERNATIONAL SEARCH REPORT

International application No
PCT/US95/05500

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-42, drawn to a first method of producing an immunoglobulin having a fully human variable region, the immunoglobulin produced by the method, and the first method of using the immunoglobulin for therapy or diagnosis in humans.

Group II, claim(s) 43 and 44, drawn to a second method of using the immunoglobulins, for treatment of an autoimmune disease in a mammal.

Group III, claim 45, drawn to a third method of using the immunoglobulins, for treatment of organ rejection.

Group IV, claim 46, drawn to drawn to a fourth method of using the immunoglobulins, for treatment of reperfusion ischemia in a mammal.

Group V, claims 47 and 48, drawn to a fifth method of using the immunoglobulins, for treatment of cachexia.

Group VI, claim 49, drawn to a sixth method of using the immunoglobulins, for treatment of tumor metastasis.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

each antibody needed for each treatment is a different antibody and each different antibody is the special technical feature which is different for each method. Each method requires a different antibody and therefore each group requires the use of an antibody having a different technical feature. Each method uses different antibodies which have different technical features because each antibody is different. Each antibody is different because each antibody recognizes a different antigen having a different primary, secondary and tertiary structure. The structure of each antibody variable region, the region involved in antigen recognition, is encoded by the DNA and therefore immunization with each different antigen induces a different arrangement of genomic immunoglobulin DNA, resulting in a DNA sequence unique for the antibody recognizing a specific antigen.
The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.